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L4 and cryoprotectant	18

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L3	liposome same cryoprotectant same (ph adj1 gradient)	2	L3
L2	L1 and cryoprotectant$	0	L2
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END OF SEARCH HISTORY

L5: Entry 15 of 18 File: USPT Aug 17, 1999

DOCUMENT-IDENTIFIER: US 5939096 A
TITLE: Liposome drug-loading method and composition

Abstract Text (1):
A method of stably encapsulating a weak acid drug in liposomes, at a high concentration, is disclosed. The method employs a proton shuttle mechanism involving the salt of a weak acid to generate a higher inside/lower outside pH gradient. The weak acid compound accumulates in liposomes in response to this gradient, and may be retained in the liposomes by cation-promoted precipitation or low permeability across the liposome transmembrane barrier. Also disclosed is a reagent combination for practicing the method, and a liposome composition formed by the method.

Brief Summary Text (38):
In the case of ionizable hydrophilic or amphipathic drugs, even greater drug-loading efficiency can be achieved by loading the drug into liposomes against a transmembrane pH gradient (Nichols, et al., 1976; Cramer, et al., 1977). Typically the drug contains an ionizable amine group, and is loaded by adding it to a suspension of liposomes prepared to have a lower inside/higher outside pH gradient. Although high drug loading can be achieved by this approach (e.g., U.S. Pat. No. 5,077,056), the drug tends to leak out over time as the liposome transmembrane proton gradient decays.

Brief Summary Text (39):
The latter problem has been addressed, for drugs having an ionizable amine group, by loading the drug across an ammonium ion gradient (Haran, et al., 1993). Ammonium ions within the liposomes are in equilibrium with ammonia, which is freely permeable through the liposome membrane, and protons, which therefore accumulate as ammonia is lost from the liposomes, leading to a lower inside/higher outside pH gradient. After establishing the gradient, excess ammonium ions within the liposomes provide a reservoir of protons, to maintain the liposome pH gradient over time. This approach, however, is limited to drugs which are positively charged in their ionized state.

Brief Summary Text (42):
The present invention includes, in one aspect, a method of forming liposomes having a higher inside/lower outside pH gradient. The gradient is established by preparing a suspension of liposomes in an aqueous solution containing a salt of a weak acid which is capable of freely permeating the liposome membrane. The suspension is then treated to produce a higher inside/lower outside concentration gradient of the weak acid. The weak acid is allowed to distribute between inner and outer compartments, acting as an inside-to-outside proton shuttle to generate a higher inside/lower outside pH gradient.

Brief Summary Text (44):
In a related aspect, the invention provides a method for loading a weak-acid compound into liposomes having a higher inside/lower outside pH gradient. Loading is carried out by adding the weak acid compound to a suspension of liposomes having a higher inside/lower outside gradient of a salt of a weak acid which includes the given cation. The protonated form of the weak acid salt acts as an inside-to-

outside proton shuttle to generate a higher inside/lower outside pH gradient to drive loading of the weak acid compound into the liposome interior.

Drawing Description Text (5):
FIG. 3 is a schematic illustration of the loading of a weak acid drug, "D--COOH", into liposomes against a higher inside/lower outside pH gradient established by the method of the present invention;

Detailed Description Text (12):
A "higher inside/lower outside pH gradient" refers to a transmembrane pH gradient between the interior of liposomes (higher pH) and the external medium (lower pH) in which the liposomes are suspended. Typically, the interior liposome pH is at least 1 pH unit greater than the external medium pH, and preferably 2-4 units greater.

Detailed Description Text (13):
II. Preparation of pH Gradient Liposomes

Detailed Description Text (14):
This section describes the preparation of a suspension of liposomes having a higher inside/lower outside pH gradient, in accordance with the invention.

Detailed Description Text (33):
C. Formation of Liposome pH Gradient

Detailed Description Text (36):
The solute species for external-medium osmolality is preferably either the salt of a strong acid, e.g., physiological saline, or a mono- or di-saccharide, such as sucrose, glucose, or mannitol. The latter type of solute is preferred where it is desired to store the liposomes by lyophilization, in which case the saccharide functions as a cryoprotectant to minimize liposome damage during freezing and rehydration.

Detailed Description Text (37):
After adjusting the external medium to produce a higher inside/lower outside concentration gradient of the weak acid salt, the weak acid is allowed to distribute between inner and outer liposome compartments, with the weak acid salt acting as an inside-to-outside proton shuttle, until an equilibrium higher inside/lower outside pH gradient is formed.

Detailed Description Text (38):
FIG. 1 illustrates the proton-shuttle mechanism by which the pH gradient is formed. The figure shows a liposome 10 having a bilayer membrane 12 and having encapsulated therein, the salt of a weak acid, in this case, the calcium salt of acetate, with the acetate anion being in equilibrium with the uncharged (protonated) form of the acid. The bilayer membrane serves as a partition between the liposome inner compartment, indicated at 14, and an outer bulk phase suspension medium 16.

Detailed Description Text (49):
It will be appreciated from the above that the pH gradient across the liposomes is self-regulating and self-sustaining, i.e., not degraded by leakage of protons into the liposomes or hydroxyl ions out of the liposomes after the gradient is formed. This feature can be appreciated from the proton shuttle mechanism illustrated in FIG. 1. Here it is assumed that a pH gradient has been established and the liposomes are stored over an extended period in suspension. During storage, as hydroxyl ions leak out from the liposomes into the external medium, and as protons leak into the liposomes from the external medium, the equilibrium between charged and protonated form of the weak acid (acetate) in the liposomes shifts toward the protonated form, increasing the level of proton shuttling out of the liposomes, acting to restore the pH gradient.

Detailed Description Text (51):
The pH gradient liposomes formed as above are used in loading a weak-acid compound into the liposomes, according to another aspect of the invention. In this method, the compound is added to a suspension of the pH gradient liposomes, and the suspension is treated under conditions effective to load weak acid compound within the liposomes.

Detailed Description Text (53):
FIG. 3 illustrates the mechanism of drug loading into liposomes, in accordance with the method. The figure shows a liposome 18 having a bilayer membrane 20 and a higher inside/lower outside pH gradient, by virtue of a higher inside/lower outside gradient of a weak acid, i.e., the anion of the weak acid, in this case the acetate anion.

Detailed Description Text (54):
The bottom of the figure shows the mechanism by which a higher inside/lower outside pH gradient is formed, as described in Section II. The upper part of the figure shows the mechanism of loading of the weak acid compound, indicated as "D--COOH". The compound, which is present originally only in the external compartment, is shown in equilibrium in this compartment between negatively charged and uncharged, protonated forms, with lower pH favoring the latter form. As indicated, the compound is able to pass through the liposome membrane only in its protonated form. In the absence of a pH gradient, the compound would equilibrate to equal concentrations on both sides of the membrane. Because of the higher internal pH, the equilibrium between the charged and uncharged form of the compound is shifted toward the charged, nonpermeable form, leading to net loading of the compound in the liposomes. Assuming the compound remains in solution in its liposome-loaded form, the extent of liposome loading for weak acid compounds is governed by the Henderson-Hasselbach relationship:

Detailed Description Text (57):
By way of example, with a pH gradient of 4 pH units, and an outside-to-inside volume ratio of 100:1, a theoretical loading factor of 100:1 inside:outside is possible. Based on these considerations alone, it will be appreciated that it is possible to achieve substantially 100% loading efficiency, i.e., loading of substantially all of the compound present into the liposomes, by proper selection of the initial external concentration of compound in relation to the known inside/outside volume ratio of the liposomes, which can be estimated.

Detailed Description Text (63):
Liposomes having a higher inside/lower outside pH gradient in response to a transmembrane difference in acetate ion concentration are prepared as described above. Typically, weak acid compounds to be loaded are added to the bulk medium at concentrations ranging from 1 .mu.M-100 mM, with the concentration selected depending upon both the absolute quantity of drug intended for encapsulation and the degree of loading efficiency desired, as discussed above.

Detailed Description Text (64):
After adding the weak acid compound to the liposomes, the liposomes are treated under conditions effective to trap the compound within the liposomes. Conditions suitable for compound loading are those which (i) allow diffusion of the weak acid compound, with such in an uncharged form, into the liposomes, (ii) lead to a desired final loading concentration and efficiency, and (iii) provide a self-sustaining pH gradient after drug loading.

Detailed Description Text (65):
Considering the first of these requirements, the loading period may range from 1 minute to several hours, and is typically between 15-120 minutes, depending on permeability of the weak acid drug into the liposomes, temperature, and the relative concentrations of liposome lipid and drug. Where the compound is one which

readily permeates the liposome membrane only above the phase transition temperature of the liposome lipids, e.g., 50.degree. C., the loading is carried out above this temperature. After loading, the liposomes are cooled below the phase transition temperature, e.g., to a storage temperature between 4-24.degree. C., such cooling acting to retard efflux of the loaded compound from the liposomes, independent of a pH gradient mechanism.

Detailed Description Text (66):
The final drug loading concentration and loading efficiency may be approximated from the Henderson-Hasselbach relationship, as discussed above. In addition to the considerations already discussed, the concentration of weak acid remaining after drug loading must be sufficient to maintain a high inside/low outside concentration gradient of the weak acid, preferably a ratio of at least 10:1. Thus, for example, if the initial concentration of weak acid is 150 mM, and the final concentration of loaded weak-acid compound is 50 mM, the final concentration of weak acid in the liposomes would be 100 mM (ignoring the loss of weak acid used in establishing the pH gradient), since capture of each molecule of compound within the liposomes, by deprotonating the compound, requires the shuttling of one proton out of the liposomes, and thus the efflux of one molecule of the weak acid from the liposomes. Assuming a V.sub.o /V.sub.i ratio of 50, concentration gradient of weak acid after drug loading in this example would be 100:50/50, or 100:1, sufficient to maintain a inside to outside drug loading ratio of 100:1.

Detailed Description Text (67):
In addition, the excess weak acid in the liposomes after drug loading provides a reservoir for sustaining the pH gradient across the liposomes over an extended storage time, as the equilibrium between protonated and unprotonated forms of the encapsulated weak acid is shifted in response to hydroxyl ion efflux or proton influx over time, as described in Section II. Accordingly, drug efflux from the liposomes on storage is effectively uncoupled from proton influx or hydroxyl-ion efflux, allowing for stable compound storage in suspension form over an extended period.

Detailed Description Text (69):
To illustrate one embodiment of the present invention, two exemplary weak acids were loaded into pH gradient liposomes. The compounds were loaded using as a driving force the pH gradient generated by a transmembrane difference in acetate concentrations, as described in Example 3. The model compounds selected for loading, 5(6)-carboxyfluorescein and nalidixic acid, are both fluorescent weak acid compounds. Their fluorescent properties provided a useful means for determining the concentration of the compounds in liposomal media.

Detailed Description Text (80):
However, with proper selection of liposome concentration, external concentration of added compound, and the pH gradient, essentially all of the weak acid compound may be loaded into the liposomes. For example, with a pH gradient of 2-3 units (or the potential of such a gradient employing an acetate ion gradient), the final internal:external concentration of drug will be about 1000:1. Knowing the calculated internal liposome volume, and the maximum concentration of loaded drug, one can then select an amount of drug in the external medium which leads to substantially complete loading into the liposomes.

Detailed Description Text (101):
In the most general embodiment, the retaining means includes the weak-acid transmembrane gradient which is due to an excess of weak acid species in the liposomes after drug loading, and which provides a reservoir for sustaining the pH gradient, as discussed above.

Detailed Description Text (103):
Finally, where the loaded compound is one which has a low solubility in the

presence of a selected cation, the cation itself provides retaining means by holding loading compound in a precipitated form that prevents efflux from the liposomes. In particular, it will be appreciated that the precipitating mechanism allows higher amounts of compound to be loaded stably into liposomes than is possible by a pH gradient alone, since the Henderson-Hasselbach relationship applies only to the solute form of the compound. For example, if the loaded compound precipitated above 5 mM compound concentration in the liposomes, a gradient effective to load to just above this relatively low concentration would be effective to load the liposomes to a high total compound concentration, e.g., 100-200 mM.

Detailed Description Text (107):
It will be appreciated how the features of the invention contribute to its applications in drug-delivery or other uses of compound-loaded liposomes. The weak-acid gradient liposomes used for compound loading are effective to generate their own pH gradient, and self-sustain this gradient by the shifting equilibrium between protonated and non-protonated forms of the encapsulated weak acid.

Detailed Description Text (133):
Two weak acid compounds, 5(6)-carboxyfluorescein and nalidixic acid, were selected for remote loading into pH gradient liposomes. Properties of the weak acids are given in Table II below.

Other Reference Publication (10):
Deamer, D., et al. "The Response of Fluorescent Amines to ph-Gradients Across Liposome Membranes," Biochem et Biophysica Acta 274: 323-335 (1972).

Other Reference Publication (16):
Nichols, J., et al., "Catecholamine Update and Concentration By Liposomes Maintaining pH Gradients," Biochim. Biophys. Acta 455: 269-271 (1976).

CLAIMS:

1. A method of forming liposomes having a higher inside/lower outside pH gradient, comprising:

preparing a suspension of liposomes in an aqueous solution of a weak acid salt comprising (i) an anion, which, in protonated form, is uncharged and is capable of freely permeating the transmembrane barrier of liposomes, and (ii) a counterion that is substantially lipid membrane impermeable,

adjusting the concentration of weak acid salt present in the external medium to produce a higher inside/lower outside concentration gradient of the weak acid salt, and

allowing the weak acid to distribute itself between inner and outer liposome compartments, with the weak acid acting as an inside-to-outside proton shuttle, thereby generating a higher inside/lower outside pH gradient.

6. A method of loading a weak-acid compound into liposomes, comprising:

adding the compound to a suspension of liposomes having a higher inside/lower outside gradient of a weak acid salt comprising (a) an anion, which, in protonated form is uncharged and is capable of readily permeating the transmembrane barrier of the liposomes, and (b) a counterion that is substantially lipid membrane impermeable, wherein the weak acid acts as an inside-to-outside proton shuttle to generate a higher inside/lower outside pH gradient and an accumulation of the compound within the liposomes, and

by said adding, achieving uptake of the compound within the liposomes.

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L1	sphingomyelin same (ph adj1 gradient)	10	L1

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L1: Entry 9 of 10 File: USPT Aug 6, 1996

DOCUMENT-IDENTIFIER: US 5543152 A
TITLE: Sphingosomes for enhanced drug delivery

Brief Summary Text (9):
In other embodiments the invention provides liposomes for delivery of a lipophilic therapeutic compound which are produced by the process of forming a liposome from a mixture which comprises sphingomyelin and cholesterol in a first buffered aqueous solution having an acidic pH greater than pH 2. The liposome is then suspended in a second buffered solution having a pH which is greater than that of the first buffered aqueous solution, thereby forming a transmembrane pH gradient which facilitates the transfer of the therapeutic compound to the liposome. In some embodiments other passive means of drug entrapment at a low intraliposomal pH can also be used in the process. These alternative processes are typically associated with a less efficient drug entrapment of drug and therefore an additional step of separating the liposome from the second buffer containing free drug may be necessary.

Detailed Description Text (7):
A representative method for producing the liposomes of the invention is now described, although it will be understood that the procedure can be subjected to modifications in various aspects without affecting the outcome. As described more fully below in the experimental section, liposomes are prepared which are able to entrap lipophilic cationic drugs in response to transmembrane pH gradients, yet which liposomes are resistant to drug leakage in the circulation. However, procedures for passive entrapment of drugs other than use of the pH transmembrane gradient can be used. Initially, liposomes containing sphingomyelin and cholesterol are prepared according to the desired molar ratio of sphingomyelin and cholesterol, e.g., 55/45 mol./mol., respectively. An appropriate buffer for formation of the liposome, and thus for forming the liposomal interior, is one which is physiologically acceptable and having an acid pH, typically about pH 2 to about pH 6, more preferably about pH 3 to pH 5, and most preferably at about pH 4. An example of an appropriate entrapment buffer is citrate buffer, adjusted to approximately pH 4.

Detailed Description Text (36):
Thus, from this Example it can be seen that liposomes composed of SM/Chol had circulation lifetimes slightly longer than similar DSPC/Chol liposomes, both in the presence and absence of entrapped vincristine (FIG. 2). SM/Chol liposomes were dramatically better than DSPC/Chol liposomes at retaining vincristine that had been encapsulated using the transmembrane pH gradient method (FIG. 4). The addition of PEG-PE to SM/Chol liposomes significantly increased the circulation longevity of the liposomes, but PEG-PE also caused a significant increase in the leakage of vincristine from the liposomes. The increased levels of vincristine remaining in circulation in SM/Chol and SM/Chol/PEG-PE liposomal formulations (FIG. 4) was a consequence of both improved drug retention in SM-containing liposomes (FIG. 3) and the increased circulation longevity of SM/Chol/PEG-PE liposomes (FIG. 2b). However, the increased circulation lifetimes of SM/Chol/PEG-PE liposomes were balanced by the lower drug retention by liposomes containing PEG-PE. Therefore, in SM-based liposomal formulations of vincristine, there was no improvement in vincristine circulation longevity by the addition of the lipid PEG-DSPE (FIG. 4). Furthermore,

since there was no improvement in vincristine retention in vivo by the use of a ph.sub.i =2.0, the optimal vincristine retention in circulation was achieved with a relatively simple liposomal formulation comprised of only sphingomyelin, cholesterol and citrate buffer (pH 4.0).

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L2	L1 and cryoprotectant	2	L2
L1	liposome same gradient same methylamine	14	L1

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